Rec'd PCT/PTO 24 MAR 2005
10/529189
PCT/US2003/030465

Materials and Methods for Diagnosis and Staging of Bovine Viral Diarrhea Virus (BVDV)

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This application claims the benefit of US provisional application, 60/413,496, filed September 25, 2002, the entire contents of which are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to the field of molecular biology and virology. More specifically, the present invention provides materials and methods for Diagnosis and Staging of Bovine Viral Diarrhea Virus (BVDV).

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these citations is incorporated by reference herein.

Bovine viral diarrhea virus (BVDV) costs the United States cattle industry more than 400 million dollars per year. The pathogenesis of BVDV infection has features that are unique to this virus and vary with the time of infection, virulence of the viral strain, and age of the animals at the time of infection.

When the infection occurs after 120 days of

gestation (post-development of the immune system) or after birth, including adult animals, the infection is referred to as acute infection. The clinical manifestation of acute infections with BVDV range from sub-clinical or unapparent infections to embryonic death, abortions, stillborn, malformed or slow growing calves. Certain strains of BVDV can cause a hemorrhagic syndrome with high morbidity and moderate mortality in adult animals. Acutely infected animals usually recover and eliminate the virus within 10 to 14 days post infection. Animals vaccinated with modified live vaccines against BVDV have an immune response similar to the one induced by natural infection.

In contrast, infection of the fetus during the first 120 days of gestation, when the immune system has not yet developed, can lead to the generation of persistently infected (PI) calves. Some of these PI calves die soon after birth, but others live for relatively long periods of time without showing any clinical signs. PI animals cannot eliminate the infecting BVDV from their system, and continuously release high amounts of virus in their bodily secretions and excretions, making them a continuous source of infection within the herd and potentially to other herds as well. Furthermore, nursing PI calves can acutely infect their mothers and other normal nursing calves, which in turn infect their own mothers while they are pregnant, producing a new cycle of infection and eventually more PI calves.

Mucosal disease, an uncommon but fatal complication observed in PI calves, occurs when the virus mutates or the animal is superinfected with an antigenically related BVDV virus. Current vaccines are relatively inefficient in preventing fetal infections, therefore the identification and elimination of PI animals is essential to any successful program for control or eradication of

BVDV.

Currently available tests for the detection of PI animals are based on the identification of the viral antigen in a blood or tissue sample (most commonly a skin biopsy) using detection methods that depend on the specific binding of anti-BVDV antibodies. Although these tests are widely used for the detection of PI animals they frequently fail to identify all infected animals (false negatives) resulting in the failure to remove all PI animals from the infected herd.

In addition, currently available tests are unable to consistently and unequivocally distinguish between persistent and acute BVDV infections requiring retesting to confirm the status of suspected PI animals. This pernicious delay in the removal of PI animals contributes to the perpetuation of the BVDV infectious cycle. Serological tests cannot differentiate between PIs and uninfected animals, or between acutely infected and vaccinated animals.

Identification and elimination of PI animals from an affected herd is the most cost effective measure to control and eradicate BVDV, underscoring the criticality of an inexpensive and convenient diagnostic test. It is an object of the invention to provide such a test and kit for performing the same.

SUMMARY OF THE INVENTION

In accordance with the present invention, methods and compositions for diagnosis of Bovine Viral Diarrhea Virus (BVDV) are provided. Specifically, a simple, convenient test for accurately diagnosing BVDV is provided. The instant method differentiates persistent BVDV infection (PI) from acute infection, and allows the skilled person to distinguish acutely infected animals from vaccinated animals. Such differentiation may be

accomplished by detecting altered expression levels of one or more markers shown in Tables 2A-B and 3A-B, or the proteins or peptide fragments encoded thereby. Most preferably, the test can be easily conducted in the field by veterinarians or cattle producers.

In one aspect of the invention, methods for detecting a BVDV surrogate marker are provided. A BVDV surrogate marker may be a nucleic acid or a protein.

In one embodiment, a BVDV surrogate marker is a nucleic acid molecule, and means of detecting the BVDV surrogate nucleic acid sequence (e.g. an mRNA which is differentially expressed in BVDV infected animals) in a biological sample are provided. Exemplary methods comprise mRNA analysis, for example by RT-PCR. In a preferred embodiment, subtraction libraries are used to determine surrogate BVDV markers which allow one to differentiate persistent BVDV infection from acute BVDV infection, and acutely infected animals from vaccinated animals. Exemplary BVDV nucleic acids are detailed in Tables 2A-B and 3A-B.

In another embodiment, a BVDV surrogate marker is a protein or peptide fragment, and means of detecting the BVDV surrogate marker protein or peptide fragment (e.g. a protein or peptide fragment which is differentially expressed in BVDV infected animals) in a biological sample are provided. BVDV surrogate proteins or peptide fragments may be detected by immunological methods, for example contacting a sample with a detectably labeled antibody immunologically specific for a surrogate BVDV protein or peptide fragment, and determining the presence of the surrogate BVDV protein or peptide fragment as a function of the amount of detectably labeled antibody bound by the sample relative to control cells.

Another embodiment of the invention comprises an isolated, enriched, or purified surrogate BVDV nucleic

acid molecule, or a nucleic acid molecule encoding a surrogate BVDV protein. A surrogate BVDV nucleic acid molecule, or nucleic acid molecule encoding a surrogate BVDV protein includes any nucleic acid molecule which is a variant or derivative of a surrogate BVDV nucleic acid or of a nucleic acid encoding a surrogate BVDV protein, but which still retains BVDV specificity. Exemplary surrogate BVDV nucleic acids are detailed in Tables 2A-B and 3A-B.

Also provided in accordance with the invention are oligonucleotides, including probes and primers, that specifically hybridize with the nucleic acid sequences set forth above.

In a further aspect of the invention, recombinant DNA molecules comprising the nucleic acid molecules set forth above, operably linked to a vector are provided. The invention also encompasses host cells comprising a vector encoding a surrogate BVDV protein.

One embodiment of the invention comprises an isolated, enriched, or purified surrogate BVDV protein or peptide fragment. A surrogate BVDV protein is intended to encompass any protein, peptide, or polypeptide product which is differentially expressed in BVDV, including acute BVDV and PI BVDV. A surrogate BVDV protein also includes any protein, peptide, or polypeptide which is a variant or derivative of the surrogate BVDV protein, which retains specificity for BVDV infected animals. Exemplary nucleic acids which may encode surrogate BVDV proteins are detailed in Tables 2A-B and 3A-B.

In another aspect of the invention, an antibody immunologically specific for a surrogate BVDV protein or peptide fragment is provided. Such antibodies may be monoclonal or polyclonal, and include recombinant, chimerized, humanized, antigen binding fragments of such antibodies, and anti-idiotypic antibodies. Exemplary

nucleic acids which encode surrogate BVDV proteins which can be bound by antibodies specific for surrogate BVDV proteins are provided in Tables 2A-B and 3A-B.

In a further aspect of the invention, kits for detection or diagnosis of BVDV are provided. An exemplary kit comprises means of detecting a surrogate BVDV expression product, including a protein, polynucleotide, or antibody. The kits may also include a pharmaceutically acceptable carrier and/or excipient, a suitable container, and instructions for administration. In a preferred embodiment, the kit comprises a test which is suitable for use in the field.

Also within the scope of the present invention, is a test for the diagnosis of infectious diseases in humans, by detection of a surrogate marker (rather than a viral antigen). Currently, all available tests, including some for which the handling of samples containing infectious virus is biohazardous (e.g. HIV, Ebola virus, etc.), are based on the detection of the viral antigen instead of a surrogate marker. A test based on a surrogate marker, which is an endogenous marker of infection, rather than the detection of the virus itself would help overcome the failure to diagnose the infection due to antigenic variation that could potentially be undetected by currently available tests and reagents.

Also encompassed within the present invention is a test for the diagnosis of infectious diseases in companion animals by detection of an surrogate marker (rather than a viral antigen). An example of a potential application would be the diagnosis of feline infectious peritonitis of cats and latent viral infections caused by herpes viruses for which current diagnostic tests based on isolation and characterization of the virus have a marginal reliability. In addition, this technology could also be used for the diagnosis of cancer through the

identification of surrogate markers (differentially expressed cancer-associated genes.)

Finally, a combination test is also provided which detects the presence of BVDV, along with a BVDV surrogate marker, to differentially diagnose BVDV infection. Likewise, several surrogate markers, or a profile of markers may be detected in combination with BVDV itself. Preferably, the instant test will be combined with a currently available test which detects BVDV protein or mRNA, to improve diagnostic accuracy of current BVDV tests.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a diagram of the BVDV pathogenesis pathway in the persistently infected (PI) calf, from in utero infection, to immune tolerance, to mutation, superinfection, and or mucosal disease, and the pathogenesis pathway of acute infection (AI).

Figure 2 depicts the experimental design utilized for subtractive libraries generated from maternal and fetal blood following experimentally induced infection with BVDV.

Figure 3 shows a restriction digest of cDNA samples by RsaI. Lanes: Lane 1 contains a 1 kb Marker. Lane 2 contains acutely infected (AI) driver cDNA. Lane 3 contains persistently infected (PI) tester cDNA. Lane 4 contains RsaI digested AI cDNA. Lane 5 contains RsaI digested PI cDNA.

Figure 4 depicts primary and secondary PCR. Primary PCR samples were run for 27 cycles for acute infection and 29 cycles for persistent infection, and secondary PCR

samples were run for 10 cycles. Lane 1 contains a 1 kb. marker. Lane 2 contains amplified cDNA from a primary PCR reaction using mRNA from acutely infected cattle. Lane 3 contains amplified cDNA from a primary PCR reaction using mRNA from persistently infected cattle. Lane 4 contains amplified cDNA from a secondary PCR reaction using mRNA from acutely infected cattle. Lane 5 contains amplified cDNA from a secondary PCR reaction using mRNA from persistently infected cattle. Lane 6 contains amplified cDNA from unsubtracted PCR using mRNA from acutely infected cattle. Lane 7 contains amplified cDNA from unsubtracted PCR using mRNA from persistently infected cattle. Lane 8 contains a 1 kb. marker.

Figure 5 shows one of six plates from a PI-specific subtracted library which were subjected to differential screening analysis using PI-specific (PI) and acute-specific (Acute) subtracted probes. Shown here is one of the plates, which illustrates identification of several cDNA-mRNAs that are specific to cells in the blood from the tester animals. Note specifically that clone B7 shows PI-specific expression. Several other clones also are specific to the PI animals.

Figure 6 shows an exemplary lateral flow system which may be used to detect BVDV surrogate markers. The control line is on the left and the test line is on the right. The sample flows laterally by capillary action past immobilized gold-conjugated antibody. The BVDV surrogate marker is detected by the formation of a visible complex. Samples 831 and 117 are positive, and Samples 87 and 1747 are negative.

Figure 7 is a chart which shows details of the clones which are differentially expressed in an acutely infected

and persistently infected BVDV cattle.

DETAILED DESCRIPTION OF THE INVENTION

Bovine viral diarrhea virus (BVDV) provides a challenge to cattle producers, because BVDV is a contagious and potentially lethal disease that is currently difficult and expensive to differentially diagnose. Provided herein is a simple and effective test for diagnosing BVDV, and differentiating persistent infection (PI) from acute infection (AI), and acutely infected animals from vaccinated animals.

This invention is based on experimental evidence that indicates that the pattern of gene expression in normal/acutely infected/vaccinated/and PI animals is different, and therefore the differential expression of genes can be used as a diagnostic marker for BVDV infection. Genes that are differentially expressed in the cells of the blood or the skin of persistently infected animals (surrogate markers) are identified using a subtraction library made between the mRNA of PI and acutely infected animals. Antibodies produced against such surrogate markers are used to develop a diagnostic test to detect PI animals, by analyzing the presence of the surrogate marker in an animal's blood or skin sample.

Current tests are performed on samples collected from young calves after birth, and therefore re-infection of the pregnant dam helps maintain the infectious cycle. Early identification of cows that are carrying persistently infected fetuses (e.g. on day 160 of pregnancy) is critical for eradication of this disease. Thus, subtractive libraries developed from blood cells collected from cows that are carrying acutely infected versus persistently infected fetuses hereby identifies BVDV surrogate markers, which allows for diagnosis of infected calves prior to birth.

I. Definitions

The following definitions are provided to facilitate an understanding of the present invention:

The term "surrogate marker" or infection marker is a marker which is differentially expressed in animals infected with a pathological condition, such as a virus. Specifically, a surrogate marker may be any gene expression product which is differentially expressed in acutely infected animals, persistently infected animals, vaccinated animals, and normal animals. A surrogate marker can be a polynucleotide, a protein, a peptide, or any gene expression product, but is preferably an mRNA or protein expression product.

A "BVDV surrogate marker" refers to a marker which is differentially expressed in animals infected with BVDV. Specifically, a BVDV surrogate marker may be any gene expression product which is differentially expressed in any or all of acutely infected BVDV animals, persistently infected BVDV animals, vaccinated BVDV animals, and normal animals. A surrogate marker can be a polynucleotide, a protein or peptide, or any gene expression product, but is preferably an mRNA or protein expression product.

A "BVDV surrogate marker profile" is an expression pattern of surrogate BVDV markers which correlates specifically to acute BVDV infection, persistent BVDV infection, BVDV vaccinated cattle, or non-BVDV infected cattle.

A "sample" or "patient sample" or "biological sample" generally refers to a sample which may be tested for a particular molecule, preferably a surrogate BVDV marker, including one or more surrogate BVDV polynucleotide, polypeptide, or antibody. Samples may include but are not limited to blood or skin, serum,

plasma, urine, saliva, and the like. Most preferably, the sample is a skin sample or a blood sample from cattle.

"Blood" includes but is not limited to whole blood, blood treated or mixed with anticoagulants, and any component of whole blood, including but not limited to serum, plasma, buffy coat, and purified peripheral blood mononuclear cells.

A "ruminant" is an even-toed, herbivorous, ungulate mammal (Order Artiodactyla) that chews cud (ruminate) and has a complex, usually four-chambered stomach containing micro-organisms that break down cellulose. Ruminants include but are not limited to cattle, sheep, antelope, deer, giraffes, elk, moose, caribou, and yak.

The term "cattle" as used herein includes any of numerous types of domestic quadrupeds held as property or raised for use, such as livestock, cows, bulls, bovine, steer, oxen, bison, and the like. The term "cattle" generally refers to multiple animals, but may also describe a single animal.

The term "ruminant nucleic acid" or "ruminant protein" refers to a nucleic acid or protein whose sequence is of ruminant origin. Preferably, a ruminant nucleic acid or ruminant protein is of bovine origin.

A "BVDV surrogate marker detector molecule" is a molecule which facilitates detecting or quantitating a BVDV surrogate marker. A BVDV surrogate marker detector molecule can be any molecule which facilitates detection of BVDV surrogate marker, including but not limited to a probe or primer which specifically hybridizes with a BVDV surrogate marker nucleic acid, or an antibody or fragment thereof which specifically binds to a BVDV surrogate marker polypeptide or peptide fragment.

The term "differential diagnosis" refers to a diagnosis which is able to differentiate between two or

more different types of BVDV infection (for example, acute infection, persistent infection, or not infected.) This test also identifies previously vaccinated animals.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the functional and novel characteristics of the sequence.

The term "nucleic acid molecule" describes a polymer of deoxyribonucleotides (DNA) or ribonucleotides (RNA). The nucleic acid molecule may be isolated from a natural source by cDNA cloning or subtractive hybridization or synthesized manually. The nucleic acid molecule may be synthesized manually by the triester synthetic method or by using an automated DNA synthesizer.

With regard to nucleic acids used in the invention, the term "isolated nucleic acid" is sometimes employed. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism or virus from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryote or eucaryote cells. An "isolated nucleic acid molecule" may also comprise a cDNA molecule. An isolated nucleic acid molecule inserted into a vector is also sometimes referred to herein as a recombinant nucleic acid molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that

has been sufficiently separated from RNA molecules with which it would be associated in its natural state such that it exists in a "substantially pure" form.

By the use of the term "enriched" in reference to nucleic acid it is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction (2-5 fold) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that "enriched" does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment. Individual clones isolated from a cDNA library may be purified to electrophoretic homogeneity. The DNA molecules obtained from these clones can be obtained directly from total DNA or from total RNA. The cDNA clones are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection of the cells carrying the cDNA library. Thus, the process which includes the

construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximately 10^{-6}-fold purification of the native message. Thus, purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly encompassed within the invention.

The term "complementary" describes two nucleotides that can form multiple favorable interactions with one another. For example, adenine is complementary to thymine as they can form two hydrogen bonds. Similarly, guanine and cytosine are complementary since they can form three hydrogen bonds. Thus if a nucleic acid sequence contains the following sequence of bases, thymine, adenine, guanine and cytosine, a "complement" of this nucleic acid molecule would be a molecule containing adenine in the place of thymine, thymine in the place of adenine, cytosine in the place of guanine, and guanine in the place of cytosine. Because the complement can contain a nucleic acid sequence that forms optimal interactions with the parent nucleic acid molecule, such a complement can bind with high affinity to its parent molecule.

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of

single stranded nucleic acid molecules of varying complementarity are well known in the art.

For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989):

T_m = 81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C, and washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured

salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide. Oligonucleotides, which include probes and primers, can be any length from 3 nucleotides to the full length of the nucleic acid molecule, and explicitly include every possible number of contiguous nucleic acids from 3 through the full length of the polynucleotide. Preferably, oligonucleotides, which include probes and/or primers are at least about 10 nucleotides in length, more preferably at least 15 nucleotides in length, more preferably at least about 20 nucleotides in length.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides,

although it may contain fewer nucleotides. The probes herein are selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-

25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

The term "vector" relates to a single or double stranded circular nucleic acid molecule that can be transfected or transformed into cells and replicate independently or within the host cell genome. A circular double stranded nucleic acid molecule can be cut and thereby linearized upon treatment with restriction enzymes. An assortment of vectors, restriction enzymes, and the knowledge of the nucleotide sequences that are targeted by restriction enzymes are readily available to those skilled in the art. A vector of the invention includes any replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or

element. A nucleic acid molecule of the invention can be inserted into a vector by cutting the vector with restriction enzymes and ligating the two pieces together.

Many techniques are available to those skilled in the art to facilitate transformation, transfection, or transduction of the expression construct into a prokaryotic or eukaryotic organism. The terms "transformation", "transfection", and "transduction" refer to methods of inserting a nucleic acid and/or expression construct into a cell or host organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt, an electric field, or detergent, to render the host cell outer membrane or wall permeable to nucleic acid molecules of interest, microinjection, PEG-fusion, and the like.

The term "promoter element" describes a nucleotide sequence that is incorporated into a vector that, once inside an appropriate cell, can facilitate transcription factor and/or polymerase binding and subsequent transcription of portions of the vector DNA into mRNA. In one embodiment, the promoter element of the present invention precedes the 5' end of the BVDV surrogate marker nucleic acid molecule such that the latter is transcribed into mRNA. Host cell machinery then translates mRNA into a polypeptide.

Those skilled in the art will recognize that a nucleic acid vector can contain nucleic acid elements other than the promoter element and the BVDV surrogate marker gene nucleic acid molecule. These other nucleic acid elements include, but are not limited to, origins of replication, ribosomal binding sites, nucleic acid sequences encoding drug resistance enzymes or amino acid metabolic enzymes, and nucleic acid sequences encoding secretion signals, periplasm or peroxisome localization signals, or signals useful for polypeptide purification.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radio immunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of

the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

The term "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The terms "recombinant organism", or "transgenic organism" refer to organisms which have a new combination of genes or nucleic acid molecules. A new combination of genes or nucleic acid molecules can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. The term "organism" relates to any living being comprised of at least one cell. An organism can be as simple as one eukaryotic cell or as complex as a mammal. Therefore, the phrase "a recombinant organism" encompasses a recombinant cell, as well as eukaryotic and prokaryotic organism.

Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid residue, provided the desired properties of the polypeptide are retained. All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

Amino acid residues are identified in the present application according to the three-letter or one-letter

abbreviations in the following Table:

TABLE 1

Amino Acid	3-letter Abbreviation	1-letter Abbreviation
L-Alanine	Ala	A
L-Arginine	Arg	R
L-Asparagine	Asn	N
L-Aspartic Acid	Asp	D
L-Cysteine	Cys	C
L-Glutamine	Gln	Q
L-Glutamic Acid	Glu	E
Glycine	Gly	G
L-Histidine	His	H
L-Isoleucine	Ile	I
L-Leucine	Leu	L
L-Methionine	Met	M
L-Phenylalanine	Phe	F
L-Proline	Pro	P
L-Serine	Ser	S
L-Threonine	Thr	T
L-Tryptophan	Trp	W
L-Tyrosine	Tyr	Y
L-Valine	Val	V
L-Lysine	Lys	K

The term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into, for example, immunogenic preparations or

pharmaceutically acceptable preparations.

"Natural allelic variants", "mutants" and "derivatives" of particular sequences of amino acids refer to amino acid sequences that are closely related to a particular sequence but which may possess, either naturally or by design, changes in sequence or structure. By closely related, it is meant that at least about 75%, or 80% or 85% or 90% or 95%, and often, more than 90%, or more than 95% of the amino acids of the sequence match over the defined length of the amino acid sequence referred to using a specific SEQ ID NO.

Different "variants" of BVDV surrogate marker members exist in nature. These variants may be alleles characterized by differences in the nucleotide sequences of the gene coding for the protein, or may involve different RNA processing or post-translational modifications. The skilled person can produce variants having single or multiple amino acid substitutions, deletions, additions or replacements. These variants may include inter alia: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the BVDV surrogate marker protein, (c) variants in which one or more amino acids include a substituent group, and (d) variants in which the BVDV surrogate marker protein is fused with another peptide or polypeptide such as a fusion partner, a protein tag or other chemical moiety, that may confer useful properties to the BVDV surrogate marker protein, such as, for example, an epitope for an antibody, a polyhistidine sequence, a biotin moiety and the like. Other BVDV surrogate marker proteins of the invention include variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at the conserved or non-conserved

positions. In another embodiment, amino acid residues at non-conserved positions are substituted with conservative or non-conservative residues. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques are known to the person having ordinary skill in the art.

To the extent that allelic variations, analogues, fragments, derivatives, mutants, and modifications, including alternative nucleic acid processing forms and alternative post-translational modification forms result in derivatives of BVDV-associated proteins which are still commonly immunoreactive with BVDV, they are included within the scope of this invention.

"Mature protein" or "mature polypeptide" shall mean a polypeptide possessing the sequence of the polypeptide after any processing events that normally occur to the polypeptide during the course of its genesis, such as proteolytic processing from a polyprotein precursor. In designating the sequence or boundaries of a mature protein, the first amino acid of the mature protein sequence is designated as amino acid residue 1. As used herein, any amino acid residues associated with a mature protein not naturally found associated with that protein that precedes amino acid 1 are designated amino acid -1, -2, -3 and so on. For recombinant expression systems, a methionine initiator codon is often utilized for purposes of efficient translation. This methionine residue in the resulting polypeptide, as used herein, would be positioned at -1 relative to the mature BVDV surrogate marker protein sequence.

A low molecular weight "peptide analog" or "peptidomimetic" shall mean a natural or mutant (mutated) analog of a protein, comprising a linear or discontinuous series of fragments of that protein and which may have

one or more amino acids replaced with other amino acids and which has altered, enhanced or diminished biological activity when compared with the parent or nonmutated protein.

The term "tag," "tag sequence" or "protein tag" refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides additional utility or confers useful properties, particularly in the detection or isolation, of that sequence. Thus, for example, a homopolymer nucleic acid sequence or a nucleic acid sequence complementary to a capture oligonucleotide may be added to a primer or probe sequence to facilitate the subsequent isolation of an extension product or hybridized product. In the case of protein tags, histidine residues (e.g., 4 to 8 consecutive histidine residues) may be added to either the amino- or carboxy-terminus of a protein to facilitate protein isolation by chelating metal chromatography. Alternatively, amino acid sequences, peptides, proteins or fusion partners representing epitopes or binding determinants reactive with specific antibody molecules or other molecules (e.g., flag epitope, c-myc epitope, transmembrane epitope of the influenza A virus hemaglutinin protein, protein A, cellulose binding domain, calmodulin binding protein, maltose binding protein, chitin binding domain, glutathione S-transferase, and the like) may be added to proteins to facilitate protein isolation by procedures such as affinity or immunoaffinity chromatography. Chemical tag moieties include such molecules as biotin, which may be added to either nucleic acids or proteins and facilitates isolation or detection by interaction with avidin reagents, and the like. Numerous other tag moieties are known to, and can be envisioned by the

trained artisan, and are contemplated to be within the scope of this definition.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which in normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands and receptors and complementary nucleotide sequences. The skilled person is aware of many other examples. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a large molecule. In embodiments in which the specific binding pair comprises nucleic acid sequences, they will be of a length to hybridize to each other under conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth in vitro for many generations.

An "antibody" or "antibody molecule" is any immunoglobulin, including antibodies and fragments thereof, that binds to a specific antigen. The term includes polyclonal, monoclonal, chimeric, and bispecific antibodies. Exemplary antibody fragments, capable of binding an antigen or other binding partner, are Fab fragment consisting of the VL, VH, C1 and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2

fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

Humanized antibodies in which CDRs from a non-human source are grafted onto human framework regions, typically with alteration of some of the framework amino acid residues, to provide antibodies which are less immunogenic than the parent non-human antibodies, are also included within the present invention.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein or compound of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules. Exemplary antibodies bind to a protein or peptide fragment encoded by a nucleotide sequence set forth in Tables 2A-B and 3A-B.

A "detection reagent" or a "marker detection reagent" is any substance which has binding affinity for a BVDV specific molecule, and includes but is not limited to nucleic acid molecules with sufficient affinity to hybridize to the BVDV specific marker, probes, primers, antibodies, fragments thereof, and the like. The "detection reagent" or "marker detection reagent" may optionally be detectably labeled.

The term "detectable label" is used herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or chemical means, is indicative of the presence of a target bioentity in a test sample. Representative examples of useful detectable labels, include, but are not limited to the following: molecules or ions directly or indirectly detectable based on light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or

luminescence properties; molecules or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Included among the group of molecules indirectly detectable based on light absorbance or fluorescence, for example, are various enzymes which cause appropriate substrates to convert, e.g., from non-light absorbing to light absorbing molecules, or from non-fluorescent to fluorescent molecules.

II. Surrogate BVDV Nucleic Acid Molecules, Probes, and Primers and Methods of Preparing the Same

Encompassed by the invention are surrogate BVDV nucleic acid molecules, nucleic acid molecules which encode isolated, enriched, or purified surrogate BVDV proteins or peptides, including allelic variations, analogues, fragments, derivatives, mutants, and modifications of the same.

Surrogate BVDV nucleic acid molecules, and nucleic acid sequences encoding surrogate BVDV proteins may be isolated from appropriate biological sources using methods known in the art. In a preferred embodiment, a cDNA clone is isolated from a cDNA expression library of bovine origin. Preferably, the sample is isolated from a bovine which has been vaccinated for, or has acute, or persistent BVDV infection.

Surrogate BVDV marker polynucleotides can be any one of, or any combination of the markers shown in Tables 2A-B and 3A-B, and further may include variants which are at least about 75%, or 80% or 85% or 90% or 95%, and often, more than 90%, or more than 95% homologous to the markers shown in Tables 2A-B and 3A-B, over the full length sequence. Surrogate BVDV marker polynucleotides also may be 60% or 65% or 70% or 75% or 80% or 85% or 90% or 95% or 97% or 98% or 99% or greater than 99% homologous to

the markers shown in Tables 2A-B and 3A-B, over the full length sequence. All homology may be computed by algorithms known in the art, such as BLAST, described in Altschul et al. (1990), J. Mol. Biol. 215:403-10, or the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular). Someone of ordinary skill in the art would readily be able to determine the ideal gap open penalty and gap extension penalty for a particular nucleic acid sequence. Exemplary search parameters for use with the MPSRCH program in order to identify sequences of a desired sequence identity are as follows: gap open penalty: -16; and gap extension penalty: -4.

Degenerate variants are also encompassed by the instant invention. The degeneracy of the genetic code permits substitution of certain codons by other codons, which specify the same amino acid and hence would give rise to the same protein. The nucleic acid sequence can vary substantially since, with the exception of methionine and tryptophan, the known amino acids can be coded for by more than one codon. Thus, portions or all of the markers could be synthesized to give a nucleic acid sequence significantly different from that shown in Tables 2A-B and 3A-B. The encoded amino acid sequence thereof would, however, be preserved.

In addition, the nucleic acid sequence may comprise a nucleotide sequence which results from the addition, deletion or substitution of at least one nucleotide to the 5'-end and/or the 3'-end of one or more of the markers shown in Tables 2A-B and 3A-B, or a derivative thereof. Any nucleotide or polynucleotide may be used in this regard, provided that its addition, deletion or substitution does not alter the amino acid sequence which is encoded by the nucleotide sequence, or it still shares a region of homology with one or more of the markers

shown in Tables 2A-B and 3A-B. For example, the present invention is intended to include any nucleic acid sequence resulting from the addition of ATG as an initiation codon at the 5'-end of the surrogate BVDV marker nucleic acid sequence or its functional derivative, or from the addition of TTA, TAG or TGA as a termination codon at the 3'-end of the inventive nucleotide sequence or its derivative. Moreover, the nucleic acid molecule of the present invention may, as necessary, have restriction endonuclease recognition sites added to its 5'-end and/or 3'-end.

Such functional alterations of a given nucleic acid sequence afford an opportunity to promote secretion and/or processing of heterologous proteins encoded by foreign nucleic acid sequences fused thereto. All variations of the nucleotide sequence of the markers shown in Tables 2A-B and 3A-B and fragments thereof permitted by the genetic code are, therefore, included in this invention.

In an alternative embodiment, utilizing the sequence information provided by the cDNA sequence, genomic clones encoding a surrogate BVDV marker gene may be isolated. Alternatively, cDNA or genomic clones having homology with the markers shown in Tables 2A-B and 3A-B may be isolated from other species, such as mouse or human, using oligonucleotide probes corresponding to predetermined sequences within surrogate BVDV marker gene.

III. Surrogate BVDV Proteins (Antigens) and Methods of Making the Same

Encompassed by the invention are isolated, purified, or enriched surrogate BVDV polypeptides, including allelic variations, analogues, fragments, derivatives, mutants, and modifications of the same which are

differentially expressed in BVDV animals. Preferably, surrogate BVDV marker polypeptides include polypeptides encoded by one or more of the sequences shown in Tables 2A-B and 3A-B. Surrogate BVDV marker function is defined above, and includes increased expression in response to BVDV infection, cross-reactivity with an antibody reactive with the polypeptides encoded by one or more of the sequences shown in Tables 2A-B and 3A-B, or sharing an epitope with the same (as determined for example by immunological cross-reactivity between the two polypeptides.)

Surrogate BVDV marker polypeptides or proteins can be encoded by one or more of the sequences shown in Tables 2A-B and 3A-B, and further may include variants which are at least about 75%, or 80% or 85% or 90% or 95%, and often, more than 90%, or more than 95% homologous to the same over the full length sequence. Surrogate BVDV marker polypeptides also may be 60% or 65% or 70% or 75% or 80% or 85% or 90% or 95% or 97% or 98% or 99% or greater than 99% homologous to polypeptides encoded by one or more of the sequences shown in Tables 2A-B and 3A-B over the full length sequence. All homology may be computed by algorithms known in the art, such as BLAST, described in Altschul et al.(1990), J. Mol. Biol. 215:403-10, or the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular). Someone of ordinary skill in the art would readily be able to determine the ideal gap open penalty and gap extension penalty for a particular protein sequence. Exemplary search parameters for use with the MPSRCH program in order to identify sequences of a desired sequence identity are as follows: gap open penalty: -12; and gap extension penalty: -2.

A full-length or truncated surrogate BVDV protein of the present invention may be prepared in a variety of

ways, according to known methods. The protein may be purified from appropriate sources, e.g., transformed bacterial or animal cultured cells or tissues, by immunoaffinity purification. Additionally, the surrogate BVDV protein may be produced using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such as pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocyte lysates. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Corp., Madison, Wisconsin or Invitrogen Corp., Carlsbad, California.

The surrogate BVDV proteins produced by gene expression in a recombinant prokaryotic or eukaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein or nickel columns for isolation of recombinant proteins tagged with 6-8 histidine residues at their N-terminus or C-terminus. Alternative tags may comprise the FLAG epitope or the hemagglutinin epitope. Such methods are commonly used by skilled practitioners.

IV. Anti-Surrogate BVDV Protein Antibodies and Methods of Making the Same

The present invention also provides methods of

making and using antibodies capable of immunospecifically binding to surrogate BVDV proteins. Polyclonal antibodies directed toward surrogate BVDV proteins may be prepared according to standard methods. In a preferred embodiment, monoclonal antibodies are prepared, which react immunospecifically with the various epitopes on the surface of the surrogate BVDV protein. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols.

Purified BVDV antigens, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which also may serve as sensitive detection reagents for the various types of BVDV infection (acute, PI, vaccination reaction, and not infected). Recombinant techniques enable expression of fusion proteins containing part or all of BVDV. The surrogate BVDV protein itself, or surface proteins or antigens from the surrogate BVDV protein may be used to advantage to generate an array of monoclonal antibodies specific for various epitopes of the surrogate BVDV protein, thereby providing even greater sensitivity for detection of the surrogate BVDV protein (and thus BVDV infection) in samples.

Polyclonal or monoclonal antibodies that immunospecifically interact with BVDV antigens can be utilized for identifying and diagnosing BVDV. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules. Other uses of anti-surrogate BVDV protein antibodies are described below.

V. Methods of Using Surrogate BVDV Polynucleotides, Polypeptides, and Antibodies for Screening and Diagnostic Assays

Surrogate BVDV nucleic acids may be used for a variety of purposes in accordance with the present invention. Surrogate BVDV nucleic acids (DNA, RNA, fragments thereof, etc.), or protein-encoding DNA, RNA, or fragments thereof may be used as probes to detect the presence of surrogate BVDV nucleic acids or protein in a sample. Methods in which surrogate BVDV nucleic acids and protein-encoding nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR). Exemplary surrogate BVDV nucleic acids and nucleic acids encoding exemplary surrogate BVDV proteins or peptides are described in Tables 2A-B and 3A-B.

The surrogate BVDV nucleic acids of the invention may also be utilized as probes to identify related surrogate BVDV variants. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, BVDV surrogate marker nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to BVDV surrogate markers, thereby enabling further characterization of BVDV surrogate markers. Additionally, they may be used to identify genes encoding proteins that interact with BVDV surrogate markers (e.g., by the "interaction trap" technique - see for example Current Protocols in Molecular Biology, ed. Ausubel, F.M., et al., John Wiley & Sons, NY, 1997), which should further accelerate identification of the molecular components involved in BVDV. Finally, they may be used in assay methods to detect BVDV.

Polyclonal or monoclonal antibodies immunologically specific for proteins encoded by BVDV surrogate markers or peptide fragments thereof may be used in a variety of assays designed to detect and quantitate the protein, as well as to detect ruminant BVDV by detecting upregulation of BVDV surrogate markers. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization of BVDV specific markers in a body cell, tissue, or fluid; and (3) immunoblot analysis (e.g., dot blot, Western blot) (4) ELISA; (5) radioimmunoassay of extracts from various cells. Additionally, as described above, anti-surrogate BVDV marker protein can be used for purification of surrogate BVDV markers (e.g., affinity column purification, immunoprecipitation).

Further, assays for detecting and quantitating surrogate BVDV markers, or to detect ruminant BVDV by detecting upregulation of BVDV specific markers may be conducted on any type of biological sample where upregulation of these molecules is observed, including but not limited to body fluids (including blood, serum, plasma, milk, or saliva), any type of cell (such as skin cells, or blood cells, or endothelial cells), or body tissue.

From the foregoing discussion, it can be seen that surrogate BVDV marker nucleic acids, surrogate BVDV marker expressing vectors, surrogate BVDV marker proteins and anti- surrogate BVDV marker antibodies of the invention can be used to detect surrogate BVDV marker expression in body tissue, cells, or fluid, and alter BVDV specific marker protein expression for purposes of assessing the genetic and protein interactions involved in BVDV and induced expression.

In most embodiments for screening for surrogate BVDV mRNA, surrogate BVDV nucleic acid in the sample will

initially be amplified, e.g. using PCR, to increase the amount of the template as compared to other sequences present in the sample. This allows the target sequences to be detected with a high degree of sensitivity if they are present in the sample.

Thus any of the aforementioned techniques may be used as a diagnostic tool for detecting surrogate BVDV markers.

Further, these techniques could be used to diagnose infectious diseases in humans, by detection of a surrogate marker (rather than a viral antigen). For example, differential gene expression could be measured in HIV, Ebola, Hepatitis, and Herpes viral infections, etc. These tests are advantageous in that they are directed to detection of a theoretically harmless surrogate marker, rather than the infectious agent itself.

Such techniques could also be used to diagnose infectious diseases in companion animals by detection of a surrogate marker (rather than a viral antigen). An example of a potential application would be the diagnosis of feline infectious peritonitis of cats and latent viral infections caused by herpes viruses for which current diagnostic tests (based on isolation and characterization of the virus) have a marginal reliability. In addition, this technology could also be used for the diagnosis of cancer through the identification of surrogate cancer markers.

The instant inventive method improves upon the accuracy of current BVDV tests. A combination test, which measures both BVDV itself, and also one or more BVDV surrogate marker, to differentially diagnose BVDV infection provides superior diagnostic results in the field.

VI. Assays for Differentially Diagnosing BVDV Using Specific Surrogate Markers

In accordance with the present invention, it has been discovered that Bovine Viral Diarrhea Virus (BVDV) is correlated with increased expression levels of certain markers, including but not limited to mRNAs and proteins. Thus, these molecules may be utilized in conventional assays to differentially diagnose BVDV. The detection of one or more of these BVDV surrogate molecules in a sample is indicative of BVDV. Similarly, specific patterns of expression allow detection of acute versus persistent infection. Alternatively, the absence of these molecules in a sample indicates that a ruminant is not infected with BVDV.

In an exemplary method, a blood sample is obtained from a bovine suspected of having an acute or persistent BVDV infection. Optionally, the blood may be centrifuged through a Hypaque gradient to obtain the buffy coat. The blood or buffy coat preparation is diluted and subjected to polymerase chain reaction conditions suitable for amplification of the BVDV surrogate marker encoding mRNA. In certain applications, it may be necessary to include an agent, which lyses cells prior to performing the PCR. Such agents are well known to the skilled artisan. The reaction products are then run on a gel. An increase in BVDV surrogate marker mRNA levels relative to levels obtained from a non-infected bovine is indicative of BVDV in the animal being tested. Alternatively, an increase in BVDV surrogate markers in AI animals relative to PI animals, or in PI animals, relative to AI animals, can differentially diagnose acute infection, or persistent infection.

In an alternative method, a skin tissue sample is obtained from the bovine suspected of having acute or persistent BVDV infection. The cells are then lysed and

PCR performed. As above, an increase in BVDV surrogate marker mRNA expression levels relative to those observed in a non-BVDV infected animal being indicative of BVDV in the test animal.

It is also possible to detect BVDV using immunoassays. In an exemplary method, blood is obtained from a bovine suspected of being infected with BVDV. As above, the blood may optionally be centrifuged through a Hypaque gradient to obtain a buffy coat. The blood or buffy coat sample is diluted and at least one antibody immunologically specific for BVDV surrogate markers is added to the sample. In a preferred embodiment, the antibody is operably linked to a detectable label. Also as described above, the cells may optionally be lysed prior to contacting the sample with the antibodies immunologically specific for BVDV surrogate markers. Increased production of BVDV surrogate markers is assessed as a function of an increase in the detectable label relative to that obtained in parallel assays using blood from non-BVDV infected cow. In yet another embodiment, the blood or buffy coat preparation is serially diluted and aliquots added to a solid support. Suitable solid supports include multi-well culture dishes, blots, filter paper, and cartridges. The solid support is then contacted with the detectably labeled antibody and the amount of BVDV surrogate marker protein (e.g., a protein or peptide encoded by a nucleic acid of Tables 2A-B and 3A-B) in the animal suspected of being infected with BVDV is compared with the amount obtained from a non-AI or PI animal as a function of detectably labeled antibody binding. An increase in the BVDV surrogate marker protein level in the test animal relative to the non-AI or PI infected control animal is indicative of acute or persistent BVDV.

In another embodiment, a first antibody which binds

to a first epitope on a target protein is placed in the well of a cartridge. Whole blood, blood collected in the presence of anticoagulants (e.g. sodium citrate, heparin), plasma, or serum is placed into the well of the cartridge. The target protein, if present in the sample, is bound by the first antibody, and then migrates laterally by a wicking action, through a filter which has been sprayed with second antibody. The second antibody has affinity for a second epitope on the target protein, or alternatively for the first antibody. The second antibody is optionally labeled with a detectable label (e.g. radiolabel, gold, biotin, etc.) The second antibody localizes the antigen, and results in the appearance of a line on the filter (see figure 6). The first and second antibodies may be generated against the full length target protein, or against the N-terminal or C-terminal halves of the target protein, so that they recognize different epitopes of the target protein.

The foregoing immunoassay methods may also be applied to any type of sample, including a urine sample.

VII. Kits and Articles of Manufacture

Any of the aforementioned products or methods can be incorporated into a kit which may contain a BVDV specific polynucleotide, an oligonucleotide, a polypeptide, a peptide, an antibody, a label, marker, or reporter, a pharmaceutically acceptable carrier, a physiologically acceptable carrier, instructions for use, a container, a vessel for administration, an assay substrate, or any combination thereof.

The following materials and methods further exemplify the invention described herein and are not intended to limit the scope of the invention in any way.

Example I

Experimental Design

Design of Subtraction Libraries

-The positive Tester-PI cDNAs, libraries and probes represent those derived from blood mRNA from six-month-old calves that are persistently infected (PI) with BVDV.

-The negative Driver-Acute cDNAs libraries and probes represent those derived from blood mRNA from six-month-old calves that are acutely infected with BVDV.

Other subtraction libraries for the calf model include:

1- The positive Tester-PI cDNAs, libraries and probes represent those derived from blood mRNA from six-month-old calves that are persistently infected (PI) with BVDV.

The negative Driver-Acute cDNAs libraries and probes represent those derived from blood mRNA from six-month-old calves that are not infected with BVDV.

2- The positive Tester-PI cDNAs, libraries and probes represent those derived from skin mRNA from six-month-old calves that are persistently infected (PI) with BVDV.

The negative Driver-Acute cDNAs libraries and probes represent those derived from skin mRNA from six-month-old calves that are acutely infected with BVDV.

Materials and Methods

cDNA synthesis and RsaI digestion

Whole blood from PI and acute animals (n = 5 each) is processed to yield mRNA using standard procedures. This mRNA is amplified using the Clontech Smart amplification kit and converted to cDNA (Figure 3) and packaged in a subtractive library using Clontech's

subtractive library kit.

PCR-Select Subtraction Procedure

PCR-Select cDNA subtraction is performed in both directions and subtracted cDNA libraries are prepared. Subtractive hybridization is performed with 1 (tester-PI): 30 (driver-Acute) ratio in both the directions and subtracted cDNA pool is amplified by PCR (Figure 4).

Construction of Subtracted Library

For PI and acute-specific subtracted cDNA pools, approximately 40 ng purified PCR-amplified secondary PCR product are cloned into the pAtlas vector (PUC base vector). The white:blue colony ratio for both of the libraries was 80:20. Ninety percent of white colonies contained plasmid with insert. The libraries contain 20% glycerol and are stored at -70°C (two tubes for PI and two tubes for acute). Six 96-well plates from the tester-specific library (PI 1-6) and five plates from driver-specific library (acute) were screened using differential hybridization and subtracted probes. The 96-well plates contain randomly picked white clones from each subtracted library grown in 100 µl of LB-amp (75 µg/ml) media for 6 hours at 37°C. Inserts are PCR amplified from these plates and subjected to differential screening analysis. All of the plates-clones are treated with 20% glycerol and stored at -70°C.

Differential Screening of Subtracted Libraries

Two µl of each PCR-amplified insert (100 ng) are arrayed in 96-well format onto a nylon membrane. Two identical membranes are prepared for each 96-well plate. Each membrane is hybridized with a different ^{32}P-labelled cDNA probe: 1) subtracted PI-specific cDNA probe and 2) subtracted acute-specific cDNA probe. Results are shown

in Figure 5.

Sequencing

All of the 49 clones from PI-specific library and 11 clones from acute-specific library were sequenced using the following primers:

F1S 5'-ATG ACG CTC AAG ACG ACA GAA-3' (SEQ ID NO:1)

R1S 5'-AAA GCA GAG GTA ACA ACG CAG-3' (SEQ ID NO:2)

Clones identified that are specific to cows/calves that are persistently infected with BVDV are sequenced to determine identity using the primers listed above.

BVDV Surrogate Markers

The relevant sequence information for all of the BVDV surrogate marker nucleic acids is shown below. The markers shown are specific for Acute Infection or Persistent Infection. Tables 2A-B and 3A-B and 3A-B list the clones with their corresponding related genebank number and descriptor, as well as their assigned SEQ ID NO. More specific information regarding the similarity of the clones to the Genbank sequences is detailed in Figure 7. Specific sequence information is provided below Tables 2A-B and 3A-B and 3A-B.

Table 2A - Summary of Acute Surrogate BVDV Markers

clone name	homologous gene name	gene bank access #	SEQ ID NO
Acute_A01	Bos taurus mRNA for similar to beta actin, partial cds, clone ORCS10586	gi-28189610 dbj-AB098930.1	3
Acute_C02	Bovine pancreatic trypsin inhibitor (PTI) gene, exon 1	gi-162765 gb-M20930.1 BOVBPTIG1	4
Acute_C06	Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII), ATPase 8, ATPase 6, cytochrome oxidase subunit III (COIII), NADH dehydrogenase subunit 3 (ND3), NADH dehydrogenase subunit 4L (ND4L), NADH dehydrogenase subunit 4 (ND4), NADH dehydrogenase subunit 5 (ND5), NADH dehydrogenase subunit 6 (ND6), and cytochrome b (cytoB) genes, complete cds; mitochondrial genes for mitochondrial products.	gi-20149081 gb-AF493542.1	5
Acute_G01	Bos taurus selectin L [lymphocyte adhesion molecule 1] (SELL), mRNA	gi-27901800 ref-NM_174182.1	6

Table 2B - Summary of Novel Acute Surrogate BVDV Markers

clone name	homologous gene name	gene bank access #	SEQ ID NO
U-Acute_A04	Bos taurus clone rp42-152a4, complete sequence	gi-21903626 gb-AC096629.9	7
U-Acute_B04	Homo sapiens BAC clone RP11-326P21 from 4, complete sequence	gi-14916216 gb-AC092631.1	8
U-Acute_C05	Gorilla gorilla On3, bc11 ABC-transporter (TAP1) mRNA, partial cds	gi-1200198 gb-L76470.1 GORTAP1A	9

Table 3A - Summary of Persistent Surrogate BVDV Markers

clone name	homologous gene name	gene bank access #	SEQ ID NO
PI1_C11	Bos taurus hemoglobin, beta [beta globin] (HBB), mRNA	gi-27819607 ref-NM_173917.1	10
PI1_H09	Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII), ATPase 8, ATPase 6, cytochrome oxidase subunit III (COIII), NADH dehydrogenase subunit 3 (ND3), NADH dehydrogenase subunit 4L (ND4L), NADH dehydrogenase subunit 4 (ND4), NADH dehydrogenase subunit 5 (ND5), NADH dehydrogenase subunit 6 (ND6), and cytochrome b (cytoB) genes, complete cds; mitochondrial genes for mitochondrial products.	gi-20149081 gb-AF493542.1	11
PI2_A03	Bos taurus hemoglobin, beta [beta globin] (HBB), mRNA	gi-27819607 ref-NM_173917.1	12
PI2_B10	Bos taurus ribosomal protein L3 (Rpl3), mRNA	gi-27807286 ref-NM_174715.1	13
PI2_F06	Bos taurus MHC class II DQB precursor BoLA-DQB mRNA (BoLA-DQB*2001)	gi-4106720 gb-AF037315.1 AF037315	14
PI3_H06	Bos taurus mitochondrion, complete genome	gi-336430 gb-J01394.1 BOVMT	15
PI5_A01	CII-3=succinate-ubiquinone oxidoreductase complex II membrane-intrinsic subunit [cattle, heart, mRNA, 1289 nt]	gi-786510 gb-S74803.1 S74803	16
PI5_A02	Bos taurus alpha globin gene, allele Y	gi-6006424 emb-AJ242799.1 BTA242799	17
PI6_A09	Bos taurus mRNA for similar to 40S ribosomal protein SA (P40), partial cds, clone: ORCS12246	gi-28189772 dbj-AB099011.1	18
PI6_D02	B.taurus mRNA for mitochondrial ATP synthetase epsilon-subunit (EC 3.6.1.34)	gi-105 emb-X16978.1 BTATPE	19
PI6_D11	Bos taurus T-cell receptor CD3 epsilon chain mRNA, complete cds	gi-1263009 gb-U25687.1 BTU25687	20
PI6_D12	Bos indicus mitochondrial partial 12S rRNA gene, strain Amritmahal	gi-21530867 emb-AJ490501.1 BIN490501	21

Table 3B - Summary of Novel Persistent Surrogate BVDV Markers

clone name	homologous gene name	gene bank access #	SEQ ID NO
U-PI1_B07	Human DNA sequence from clone RP5-1071L10 on chromosome 20 Contains part of a gene for a new member of the thymosin/interferon-inducible multigene family and the 3' part of the BCAS4 gene for breast carcinoma amplified sequence 4, complete sequence.	gi-8217426 emb-AL133228.18	22
U-PI1_E02	Homo sapiens chromosome 21 PAC LLNLP704M17648Q13, complete sequence	gi-6249454 emb-AL035610.3 HSM17648	23
U-PI1_G01	Homo sapiens sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1 (SOAT1), transcript variant 688113, mRNA	gi-24431944 ref-NM_003101.2	24
U-PI2_F11	Homo sapiens ribosomal protein L35a (RPL35A), mRNA	gi-16117790 ref-NM_000996.2	25
U-PI3_E07	Arabidopsis thaliana chromosome 1 BAC F3I17 genomic sequence, complete sequence	gi-12323825 gb-AC016162.5 AC016162	26
U-PI4_A08	Homo sapiens chromosome 5 clone CTC-555C2, complete sequence	gi-11141985 gb-AC020902.6 AC020902	27
U-PI5_C02	Homo sapiens chromosome 10 clone RP11-574K11, complete sequence	gi-21535944 gb-AC022400.9	28
U-PI5_E02	Homo sapiens BAC clone RP11-814H16 from 4, complete sequence	gi-20279521 gb-AC106052.4	29
U-PI5_E07	Homo sapiens eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa (EIF2B1), mRNA	gi-4503502 ref-NM_001414.1	30
U-PI5_H06	Homo sapiens eukaryotic translation initiation factor 3, subunit 6 48kDa (EIF3S6), mRNA	gi-4503520 ref-NM_001568.1	31
U-PI6_A05	Human DNA sequence from clone RP11-205M20 on chromosome 9, complete sequence	gi-16304896 emb-AL353671.6	32
U-PI6_A06	Klebsiella pneumoniae contig region pSL029	gi-9909725 emb-AJ293849.1 KPN293849	33
U-PI6_A10	Homo sapiens chromosome 6 open reading frame 62, mRNA (cDNA clone MGC:57512 IMAGE:6499979), complete cds	gi-28839592 gb-BC047866.1	34

U-PI6_A12	Homo sapiens, similar to pleckstrin homology, Sec7 and coiled/coil domains 2 (cytohesin-2), clone MGC:41793 IMAGE:5269719, mRNA, complete cds	gi-24324831 gb-BC038713.1	35
U-PI6_B10	Bos taurus clone rp42-158g13, complete sequence	gi-20429385 gb-AC105306.13	36
U-PI6_C02	Homo sapiens sarcoma amplified sequence (SAS), mRNA	gi-21264346 ref-NM_005981.2	37
U-PI6_D06	Human DNA sequence from clone RP11-2P5 on chromosome 13, complete sequence	gi-11414487 emb-AL157877.11	38
U-PI6_D10	Homo sapiens CDW52 antigen (CAMPATH-1 antigen), mRNA (cDNA clone MGC:1783 IMAGE:3345325), complete cds	gi-12653718 gb-BC000644.1	39
U-PI6_E03	Homo sapiens, clone RP11-51J9, complete sequence	gi-22004283 gb-AC026979.9	40
U-PI6_F10	Human DNA sequence from clone RP11-496H23 on chromosome 10, complete sequence	gi-15617275 emb-AL391318.21	41
U-PI6_G06	Mus musculus peptide N-glycanase (Ngly1) gene, complete cds	gi-30517851 gb-AY225417.1	42
U-PI6_G08	Homo sapiens myotrophin (MTPN), mRNA	gi-21956644 ref-NM_145808.1	43
U-PI6_G11	Homo sapiens 3 BAC RP11-407H3 (Roswell Park Cancer Institute Human BAC Library) complete sequence	gi-19774266 gb-AC069243.16	44
U-PI6_H02	Homo sapiens BAC clone RP11-275G11 from 7, complete sequence	gi-16756340 gb-AC073635.8	45
U-PI6_H10	Homo sapiens chromosome 5 clone CTD-2134K2, complete sequence	gi-15187234 gb-AC020925.8	46

Acute_A01 (SEQ ID NO:3)

GTACGCCCTTCCCCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACGGACTACCTCATGAA
GATCCTCACGGAGCGTGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTCCGTGACATCAAGGA
GAAGCTCTGCTACGTGGCCCTGGACTTCGAGCAGGAGATGGCCACCGCGGCCTCCAGCTCCTCCCTGGA
GAAGAGCTACGAGCTTCCTGACGGGCAGGTCATCACCATCGGCAATGAGCGGTTCCGCTGCCCTGAGGC
TCTCTTCCAGCCTTCCTTCCTGGGCATGGAATCCTGCGGCATTCACGAAACTACCTTCAATTCCATCAT
GAAGTGTGACGTCGACATCCGCAAGGACCTCTACGCCAACACGGTGCTGTCCGGCGGGACCACCATGTA
CCTCGGCCGCGACCACGCTGGG

Acute_C02 (SEQ ID NO:4)

GTACGCGGGGAGGAGCCCTCCTTGCAAGGCCATGAAGTTGAGCCAGCTCTGCCTCTCCGTAGCCCTTCT
GGTCCTCCTGGGCACCCTGGCGGCCAGCACTCCAGGGTGTGACACCAGCAACCAGGCCAAAGTGCCTGA
TCACACCCCTCAGCCAGGATACTAAAAGCTCCATCCATCCTCAGCATCCTCAGGAGCCCTCCTGCGAGT
CCACGAAGATGAACCGACCCTGCCTCTCTGCAGCCCTTCTCTTCCTCCTGGTTATCCTAGTGGATGGCA
TCTCAGAGGATACTAACAAGAGCCATGACCACGGTTATGAGATGACTTACGGAAGACTAAATGAGAAGC
ATTCAGCAGCTTCTAAGCCTGCCTTCTGCCTGAAGCCTAAATCAATAGGTCCCTGCAAGGGCAGGAAGA
TCAGGTACCTCGCCGCGACCACGCTGGG

Acute_C06 (SEQ ID NO:5)

GTACTATTCTTACCGGATTTTTCTGAGCTTTCTACCACTCAAGCCTCGCCCCCACCCCTGAACTAGGCG
GCTACTGACCCCCAACAGGCATTCACCCACTAAACCCCCTAGAAGTCCCACTGCTCAACACCTCTGTCC
TATTGGCTTCCGGAGTTTCTATTACCTGAGCCCATCATAGTTTAATAGAAGGGGACCGAAAGCATATAT
TACAAGCCCTATTTATCACCATCACATTAGGAGTCTACTTCACACTACTACAAGCCTCAGAATACTATG
AAGCACCTTTTACTATCTCCGACGGAGTTTACGGCTCAACTTTTTTTGTAGCCACAGGCTTCCACGGCC
TCCACGTCATCATTGGGTCCACCTTCTTAATTGTCTGCTTCTTCCGCCAATTAAAATTTCATTTTACTT
CTAACCACCACTTCGGCTTTGAAGCCGCTGCCTGATACTGACATTTCGTAGACGTAGTCTGACTTTCCT
CTATGTTTCTATCTATTGATGAGGCTCCTAACAAAAAAAAAAAA

Acute_G01 (SEQ ID NO:6)

GCCAGGAAGGGGAGATTTCCAGTTTGGACTAGTTAAGGATTAGTTATGAAAACAGTGGCAAGGGTGGGG
GATGCCTGAGATGAATCAGAAAGAGTAGATAGAATCAAGGCGGATACGTTAAGAGGATTGAGGGTGAGG
AAGAAGGGACATTTCAACCATGAGTCAAGACATCTCTTTCCACTTGCTTTAGCTCTTCTGTATGACATC
AAAGAGATGCCTGCAGCTGCCATTCTAGTGATGAGTGGTCATAAGAGAAACATCTGGATGGGGCTAATA
AAAAAGAAACACTAAAACCCCAATCCAAAAATAATTAAAAAAATAAAACCAACAGTGCAAGCTTTTTCT
TCTCTTCTTTTGTTTGGTGCTCATGAGCTGATATATGTGGAAATGAGCCAACTGA

U-Acute_A04 (SEQ ID NO:7)

GTACGCGGGACTTAGATCTTTAATCCATGTTGAGTTTATTTTTATGTATAATGCTGAGAGTGTTCTAAT
TTCATTTGTTTACATATCAGTTCAGTCCAGTCGCTCAGTCGTGTCCAACTCTTTGCAACCCCATGAATC
GCAGCACACCAGGCCTCCCTGTCCATCACCAACTCCCGGAGTTCACACAGACTCATGTCCATCGAGTCA
GTGATGCCATCCAGCCATCTTATCCTCTGTCATCCCCTTCTCCTCCTGCCCCCAATCCCTCCCAGCATC
AGAGTCTTTTCCAATGAGTCAACTCTTCGCATGAGGTGGCCAAAGTATTGGAGTTTCAGCATCATTCCT
TCCAAAGAAATCCCAGGGCTGATCTCCTTCAAAATGGACTGCTTGGATCTCCTTTCAGTCCAAGGGACT
CTCAAGAGTCTTCTCCAACACCGCAGTTCAAAAGCATCAATCAGCTGTCAGTTTTTTCTCAGCACCACG
TATTGACAAGACTGTCTTTCTCCAT

U-Acute_B04 (SEQ ID NO:8)

GTACAAAAGATAGTCCCCAAAGTTACAGATTTATTGGCCCTCTAGGATTTGAATTTGTTTTTCTCTAAC
AAAGCATTTCAGAATGCCATCTCCAATAACTATACCAACTTCTGTTCCTCAGATGATCTTTGAATCAGT
TTAGTGATTAATGGGTTAAATACACACTCTAAGTCTGTATTTGAATGCCATGTTTTTAGCTTAAAAAAG
TTTACACTGTGATATCTTGTTCAGACCCTCCCCCCAAAAAAACCCCACGAATCCAAAGTTCAGGAATTT
CTACACAATCTTTTCCCTCTAAAAGTTTGAGTGAAGTCAAATTGGATACTCTTGATGAATGTGTTGTGC
TTGTAATTAGAACAGAACAGTAACTGTTGCTTTTTAAAAAGTAATTCAGGCCACGGCCCTCCAATGAGG
ACGGTGAGGCAAGAGAGACGGAGAAGAGGGGGCCCGGTCCAGGCGCTTCCGCTCAACCAATAGCGATCT
CAGGTCCTCGACGGCTGACCCCAGCCTCAGCTCCGACTTGGATTTCTGAACTCCCTCATTGTTAGGATC
AGCACAGACACGCTGCCCTTCTGGGTGATGAAGATG

U-Acute_C05 (SEQ ID NO:9)

GTACAGAATAAAGGTGACCAGGCGCCCACTGCTGACAGACCCACTTGTCACCAGCTGCCCGCCAAAATA
CAGGATTCCCACCTTCAGCAGCATCCCTGAGAGACTGGAGGTCCAGACGTTGACCGCGTAGGCCAGGGC
CTCCTTCTGGTTGAGCACCATCATCTCATGCAGCTTTTACCTGAACTTCTGGGCCTCACCCTCTTCATT
GGCAAAGCTCCGAACTGTAGGCATGGCTGACAGCACCTCGATGGCCACCTGGCTGGACTTTGCCAGAGA
TTCTTGTACCTCGGCCGCGACCACGCTGGG

PI1_C11 (SEQ ID NO:10)

GTACGCGGGGACACTTGCTTCTGACACAACCGTGTTCACTAGCAACTACACAAACAGACACCATGCTGA
CTGCTGAGGAGAAGGCTGCCGTCACCGCCTTTTGGGGCAAGGTGAAAGTGGATGAAGTTGGTGGTGAGG
CCCTGGGCAGGCTGCTGGTTGTCTACCCCTGGACTCAGAGGTTCTTTGAGTCCTTTGGGGACTTGTCCA
CTGCTGATGCTGTTATGAACAACCCTAAGGTGAAGGCCCATGGCAAGAAGGTGCTAGATTCCTTTAGTA
ATGGCATGAAGCATCTCGATGACCTCAAGGGCACCTTTGCTGCGCTGAGTGAGCTGCACTGTGATAAGC
TGCATGTGGATCCTGAGAACTTCAAGCTCCTGGGCAACGTGCTAGTGGTTGTGCTGGCTCGCAATTTTG
GCAAGGAATTCACCCCGGTGCTGCAGGCTGACTTTCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTGG
CCCACAGATATCATTAAGCTCCCTTTCCTGCTTTCCAGGAAAGGGTTTTTTTCATCCTCA

PI1_H09 (SEQ ID NO:11)

GTACGCGGGATGAACATTAATATTAATATCTCTGATCCTATTTATTGGATCAACAAACCTACTAGGCCT
ATTACCCCATTCATTCACACCAACAACACAACTATCAATAAACCTAGGCATAGCCATCCCCCTGTGAGC
AGGAGCCGTAATTACAGGATTCCGCAATAAAACTAAAGCATCACTTGCCCATTTCTTACCACAAGGAAC
ACCCACTCCACTAATCCCAATACTAGTAATTATTGAAACTATCAGCCTTTTTATTCAACCTATAGCCCT
CGCCGTGCGGTTAACAGCTAACATCACTGCAGGACACCTATTAATTCACCTAATCGGAGGA

PI2_A03 (SEQ ID NO:12)

GTACTTTTTTTTTTTTTTTTTTTTTTTGGAATGAAAATAAATGTCTTTATTAGGCAGAGGCCAGATGCTC
AAAACACTTCATAATTTTTCCATATTCAATCTTTGGGCTCTGAGGATGAAAAAACCTTTCCTGGAAAGC
AGGAAAGGGAGCTTAATGATATCTGTGGGCCAGGGCATTGGCCACACCAGCCACCACCTTCTGAAAGTC
AGCCTGCAGCACCGGGGTGAATTCCTTGCCAAAATTGCGAGCCAGCACAACCACTAGCACGTTGCCCAG
GAGCTTGAAGTTCTCAGGATCCACATGCATGCTTATCACAGTGCAGCTCACTCAGCGCAGCAAAGGTGC
CCTTGAGGTCATCGAG

PI2_B10 (SEQ ID NO:13)

GTACTGTCAGGTCATCCGTGTCATTGCCCACACCCAGATGCGCCTGCTTCCTCTGCGCCAGAAGAAGGC
CCACCTCATGGAGGTCCAGGTGAACGGAGGCACTGTGGCCGAGAAACTGGACTGGGCCCGTGAGAGGCT
CGAGCAGCAGGTCCCTGTGAGCCAAGTGTTTGGCCAGGATGAGATGATTGATGTCATTGGGGTGACCAA
GGGCAAAGGCTACAAAGGTGTCACCAGCCGTTGGCGCACCAAGAAGCTGCCCCGTAAGACCCACCGAGG
ACTGCGCAAGGTTGCCTGTATTGGGGCGTGGCATCCTGCCCTGGCGGCCGCTCGAGG

PI2_F06 (SEQ ID NO:14)

GTACTTTTTTTTTTTTTTTTTTTTTTTTTTCCAGGTAACTCATAATCATGTTTAATCATGATAAAAAAAT
TCTATAGCCAGGCAAATGGTTTCAATGTGCTTCTCTCCCACAGTTCTGCTCTGGAAAAATGGAAACAGA
AAAACTCTTGGGGTCTGAGTAGATGCAGCTAGAAGAGGCTTCAGGGGTCAGTGCAAGAAGCAGCCAGAT
CCTTGAAGGCAGGGGTCACAGGAAGTGACCTGATGACTGCGTCAGAGTCACTGTAGGACCTGATCTCAG
TGGAACAGGATGGCAGGCAGCTGAGAATTCTGGACAAGAACAGGCAGCTATTACAGAAGAGCAAAACCA
ATCCCCATCAAAGCATCCTCAGGA

PI3_H06 (SEQ ID NO:15)

GTACTCTGGCGAATAATTTTGTTTATGTAATTATCTGTGTTTAGGGCTAAGCATAGTGGGGTATCTAAT
CCCAGTTTGGGTCTTAGCTATAGTGCGTCGGCTATTGTAGGGTCACTTTCGTCATTTATTTTTATTTTA
ATCATGGCTTTTTACAGCTTAGTTAGAATTTAACCCTATTTGGTATGGTGCTTTAACACGTTTTACGCC
GTACCTCGGCCGCGACCACGGG

PI5_A01 (SEQ ID NO:16)

GTACTCTACTGCTGAGCTCCAAGGGGATCTGCACGCAAAGAAGGGTCTAAACAAATCAGAATAACTTTG
CCCTGGCCAAAGACAAGAGTAACAAACAGGAAGACAGGTCAATGTGATAATTTGTAGGATGATGACACT
GGGAACTTCAGCTCTTCACATGGCTGCCAGCTCTACAGAGGACAACACAGTAAGAACCAGGACAGCCAC
TCCAGACTGGTGTAGCTGGGAAATCGTCAGGCCTTTTCCTAGATCCCACATCAAGTGTCGGATCCCATT
CCAGGTGTGATACATGAGAGGGAAGACAAGTGCAAATTTGGCTGTGTGGATCAGTGCTGGCCCCAAACA
CAGGGACTTCACAAATTC

PI5_A02 (SEQ ID NO:17)

GTACGCGGGCGCGCTGACCAAAGCGGTGGAACCCTGGACGACCTGCCCGGTGCCCTGTCTGAACTGAGT
GACCTGCACGCTCACAAGCTGCGTGTGGACCCGGTCAACTTCAAGCTTCTGAGCCACTCCCTGCTGGTG
ACCCTGGCCTCCCACCTCCCCAGTGATTTCACCCCCGCGGTCCACGCCTCCCTGGACAATTCTTGGCCA
ACGTGAGCACCGTGCTGACCTCCAAATACCGTTAAGCTGGAGCCTCGGCGACCCCTACCCTGGCCTGGA
GCCCCCTTGCGCTCTGCGCACTCTCACCTCCTGATCTTTGAAT

PI6_A09 (SEQ ID NO:18)

GTACTGTTCCATTTGGAACGTCAAGGTTGGTGCCACCTAAGCTGGGTTCCTGCTGCAAGGAATTTGAGG
ACATCCTCCTCCTTCATTTGCAGGACATCAAGGGCTCCGGACATTGTGAAAGCTTCCCTTTAAGTTACG
ACGGGAATTCAAGACAACGCCGTATGGACCCCTCTCTAGGTAGCGCCGAAAGCCCCGCGTACCTCGGCC
GCGACCACGCGGG

PI6_D02 (SEQ ID NO:19)

GTACTTTTTTTTTTTTTTTTTGGTGCTGCCAGAAGTCTTCCTGGCGTTTGCTTTGAATTCAGTCTTCAGT
GCATCTCTGACTGCTTTTGCACAGATCTGGGAGTATCGGATGTAGCTGAGTCCACGCCTGTCGCCAGTT
ACCTGCCCTGGCGGCCGCTCGCGGGCGTACTCTGTGTTGTTACCACTGCCTTTGGAGCTCCATTCGCCC
TATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTTCGTGACTGGGAAAACCCTGG
CGTTTACCCAACTTACTCGCCTTGCAGCACATCCCCTTTCGC

PI6_D11 (SEQ ID NO:20)

GTACCCAATCCAGACTATGAGCCCATCCGGAAAGGCCAGCGGGACCTGTATGCTGGCCTGAATCAGAGA
GGCGTTCGACAACTCCTGACACCATCTCCCACTGACCCAGGTCTGCCTCCTCTCCAGGCCTGCCACTCC
CTGTTTGTTCCCTGGGCAAATCTTGGACCCCACAGGAGAACTGTTCCTCTGCCTTGTGGGAAGCTTCCA
CCCTCAGCCTTGTCCCCACAGCCTCCTTCCTGCCTTCTCTGCTGGCGCCCAGTCCAAGGATATTGCTGC
CTTATTATCCTTTGAAACATCACAGCTACTCACCCCTTCACACCTGGTTGGCCTTCTTGTCAGGATATT
TATTTCCGTTCTTCACCCCACCCTGCCCCCATTTGTTCCTTCCCCAGTGGATATTACTATTCCCCTTGG
TTCCCTCTTTCTCTTGCAGAGAAATTGCCCCATCCCTAACTATT

PI6_D12 (SEQ ID NO:21)

GTACTTTTTTTTTTTTTTTTTTTTTGTTGAAGATGGCGGTATATAGACTGTATTAGCAAGAATTGGTGAG
GTTTATCGGGGTTTATCGATTATAGACACAGGCTCCTCTAGAAGGATATAAAGCACCGCCAAGTCCTTT
GAGTTTTAAGCTGTTGCTAGTAGTACCTGCCCTGGCGGCCGCTCGAGGG

U-PI1_B07 (SEQ ID NO:22)

GTACATTCCACAAGCATTGCCTTCTTATTTTACTTCTTTTAGCTGTTTAACTTTGTAAGGATGCAAAGA
GGTTGTGACGAGTTTAAATGACTGTGCTACCCCTTTTCACATCAAAGAATGGAGAACTACTGACAACGT
AGGCCGCACCTGCCTCTCCCATCTGCTTGTGTGGCTGGCAGGGAAGGAAAAGAACTTGCATGTTGGTGA
AGGAGGAAGCTGGGTGGGACGACAGTGAAATCTAGAGTAAAAAGCTGGTCCAAGGTGTTCTGCGGGCTG
TAAAATGCAGTTTAATCAGAGTGCCATTTTTTTTGTTGTTCAAATGATTTTAATTATTGGAATGCACAA
TTTTTTTATTATGCAAATAAAAAGTTTTAAAACCTGAAAAAAAAAAAAAAA

U-PI1_E02 (SEQ ID NO:23)

GTACCTGTGCTTGCTTGGTTCTAAATGCCATGCTATGTATTTTCTTTTATAACAGATGTTGGTTCAAAT
TTGGGTAAAAGACAAAATCAAACTACATCTTGCTTTTAAAATATATACTGCATAAATATACTAAAGAAA
ACAAAAGTTGAAAATTTATGGTCAATAATGACTGCTTACAGAAACAGAAACAAAGGTATTATTTTCAGT
CTTAACTTTAGAAAAAATATAATTCAAGGCAAAAGTATTAGACAAAGAAAATGACTTCATTTATTTAAA
TTCTGAAATCCAAATAAAAGCATGTCATCCAAATAAAAGCATTCTTTCATGAGGCAAATATTAAAA

U-PI1_G01 (SEQ ID NO:24)

GTACACTGACAGTCCTTATTTCCTGTTTCAACGCTGGGCCAGAAGGCTATCACAGGACTTCTCACCCAG
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TTTATGTTGTGTTAGCATATACACTACCACCAGCTTCCCGGTGCATTGTTATATTAGAACAGATTCGTT
TTATAATGAAGACCCACTCATTTGTCAGAGAGAATGTGCCTCGGGTACCTGCCCTGGCGGCCGCTCGAG
GG

U-PI2_F11 (SEQ ID NO:25)

GTACATATAAGCACATCTCTTGCCTAAATAGAATTCAGTTTCATCTCGAGCATATACACCTTCAATTTT
CAGGAGAGCAGTGTGTTCCCTTTGGTTCCGTAGACCCCGTTTATAGCCAGCAAAAATGGCCTTGGACCA
CAGCCTTCCAGACATATTTGTCGTTTTAGAAGTCCCGTTCCCAGCAGGCCTTCACCCCGCGTACCTCGG
CCGCGACCACGCTGGG

U-PI3_E07 (SEQ ID NO:26)

GTACCTTCACAAGGTAATTTGTCCTTTGGTGTATAAAATATCATTTAACTAGGAAATATATTAAAAGCA
CGCTTTAATGCAGTGAGGAAATGCAGTGGATTTGACGATGAATTAGTCACTGGAGACATTCCCAACCCT
CTATGGCATTTTGTGACCAATCTTTGTTACCTAAATATTGGGTTCTCATCAAACACTTAAAAATTCACT
CCACTGAATTTAAGGACATTGTTCTAAGATGCCAATGCCACATCACTTGGAAGCTTAAGAGAAGTTCCC
CATTCCCACTGCATAAATATTCAAGACGGAACTATTTCCCTGTGAGGATTCAGCACTGCTGCTGCTGCT
GCTAAGTCGCTTCAGT

U-PI4_A08 (SEQ ID NO:27)

GTACTGTAAGTAACATGAAGTTGTAAGGGTGTTTTCAGTATTCTTGGTCTTCTGTTGTTTTTCTGTCGT
CCTCTTAACTGGTTTTTTAAAATAAAAATGAAACTGTAATCATTTTTGGTATATATTCATTAAGATTCC
TGCACCATTATGAAATATTAAAACTTGCCTCTTTAAATCACTTGTATAAGTATGAATTTTAATTTTAGT
ATTTGAAGCTTTGCTAGGTGAAGATTTCCAGCAGCTCAAAAGTTCTTATGGTCAATATTGACAGATTAA
GTAGGGAAGTCAATTTTATTTTTTTATTATTCCCACTTGCCAAAGTAGTACAGCAGCCATTAACTGAA
CTAGAGAAGAAAAAGG

U-PI5_C02 (SEQ ID NO:28)

GTACAGCAAAAGTGACGCAGTTATACACACATAAACATCTTTTTCATATTTTTTCATACTTTTCCATTA
TAGTTTGTCACAGGATATAGTCCCCTGTGCTGTTCATCCATCCTGTATATAATAGGATGTCTGTTTCTG
TGCCAGTACCTGCCCTGGCGGCCGCTCGAGGG

U-PI5_E02 (SEQ ID NO:29)

GTACTTTTGAACAACTTCTTTTTTCCCTTGGTTCATAACAGTTTAAATTCCAAACTTTTGACTTTTTTT
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AGAGATTAGAGAGTCGCTAGAAAATGGCTTGATTATAATAAATGCATTGCTCTGGTGCTCTATATAATG
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TTAATATCTAATATTA

U-PI5_E07 (SEQ ID NO:30)

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TGATATTCTCCTGAGGAAAATCTCTCCCCGCTCAATCATGATCTTTTTACATTTGGAGTAATCAGAGTA
TTCCAGGGAGGTCAGGCTGATGAAACGCAGGAAGAGCTCCCCGCCCGACGACACGGCCACAGAAGAGTC
CACGCCACACAGGGTTTCTATGGCACTGGTGAGATTCGCCCTCAGGCCCTGGATTGTCTCCCCTGTATC
TCTTCTCAAGTACCTGCCCTG

U-PI5_H06 (SEQ ID NO:31)

GTACTTTTTTTTTTTTTTTTTTTTTTTTGGAATGAAACTCTCAATTTTATTCAAATTCTCCAAAATTTAT
ACTAAATACGTTGTTTCTAAAAGGTTTCACCTTCGTATAATGGCTTTATTATTTCGCCTTTCTTTTAAA
GGTTTTTCAATTTTTCTGTACCTCGGCCGCGACCACGCTGGG

U-PI6_A05 (SEQ ID NO:32)

GTACTTTTTTTTTTTTTTTTTTTTTTTTTTGTTGAATACACATTTGCTTTACTGGCAGTCCAGATAAAC
AAGTGTATAAAGGAAATATTTAAATTTTTCTTTTGGCAGATGTCAAGATAAGCTGTGCAGATTAAAATG
CCTCAATTTAACCTGAAGAAATGGTTTTGAATCCTATAAATTTTTAAGAAGAAAAATAAAGACTGTCCA
AAGGGATTCTTCTAGCTTATAACACGTTTCCAGAATAACTCTCAAAAAGAAATTCAAAAATACTCATTG
GCTACTTTAACAATATGTCATACCATATGGCTTTACGAAATAACTTCT

U-PI6_A06 (SEQ ID NO:33)

GTACTGATAGTGAGAAATCCTGCTCTAGAAAGTGTGAGAGTATATAGAAAATGTTTAAAGGTTATTTTC
AGGAACGTTTCTAGAGTTTATACAAGTCTAGGATAAGTAGAAAAAAGCTAGAATTCAATACTGTTCTTG
CCTGTAGTTCCAAAACATGTGCTTGCCCTACTAAACCATGGTACCTGCCCTGGCGGCCGCTCGAGGG

U-PI6_A10 (SEQ ID NO:34)

GTACGCGGGACCAGGAGATGGATTTCATTCTTTGGCCTCGGAATGATATTGAAAAAATTGTCTGTCTCC
TGTTCTCTAGGTGGAAGGAATCTGATGAGCCTTTTAGGCCTGTTCAGGCCAAATTTGAGTTTCATCACG
GTGACTATGAAAAACAGTTTCTGCATGTACCTGCCCTGGCGGCCGCTCGAGGG

U-PI6_A12 (SEQ ID NO:35)

GTACCGGATCTCGGCCCCCACGCAGGAGGAGAAGGACGAGTGGATCAAGTCCATCCAGGCCGCTGTGAG
CGTGGACCCATTCTATGAGATGCTGGCAGCAAGGAAGAAGCGGATTTCAGTCAAGAAGAAGCAGGAGCA
GCCCTGACCCCTTGCCCCCCACTCCATTATTTATTTCGGAGCTGCCCTGCCCTGGCGGCCGCTCGAGGG

U-PI6_B10 (SEQ ID NO:36)

GTACATCATCTTTGGCCCTGTGGCCTGTTAACATTTTGTAACTTTTTTTTTGATAAAGGTTGGTCAACC
AGAAAAACCTGTTTTTTCTTTATCTTTTTAGTCTGTAGTCTCCCTCAGGAAACAGAGCTACATTTTTATG
GAGCAAGGGTGTAGTGGGTTACAACAAAGAAAGAATTTATTAACTCAAGGGTCTTATGTTGCTAATGCC
AAGGCTACTACCTATTTTTCTTGCATACCAACTGTACCTCGGCCGCGACCACGCTGGG

U-PI6_C02 (SEQ ID NO:37)

GTACTTGGTGGTGGTTGACAGCACCTACCAGTCCAGCAACCGCGATGAGAAGAAGGAAGACTCCCACTG
CAATGACTCCTCCGATGATATGGATGCTGGACACCAGACCCAGGCCCTTAGCCCATGCAGCCACTCCAA
TGAGCAATAAGCCTACCAGCATGTAGACCACGTTGAGCGCGCAGAGCGCATTCTTGGAGCAGGCAAAGC
CTCCGCAAACCATCTCCCCAGCTTTGGGGACCCAAGTGGTCCCAAGGACTTGGGGGGAGGGTCTCGCCG
GACCGTTCTTGGACCCCGACAGCTTCTGTCTCTTTTCCCCGCGTACCTCGGCCGC

U-PI6_D06 (SEQ ID NO:38)

GTACGCGGGTGGAGGCCTTTGCAGAGAAAGCAAACTGAGCCCCAGTGAGGATTATAAATTGGTTAGACA
AATAGGAGGAGAAATATGGAAAGTAAAATGCTCAGGCACATTGAGGAGAAGTTGGTGCTAAGAACAGAG
CTGAAGCAAGAAGAGGCAGGAGCCAGGTCAAGCAGAGGTATTTAGGCACTGCCCAAGTCTTATATACTT
GCCCATCCCTGCCTCTTGCTGCCTTCTGTTCCCCCCACAACTCTCATCCCTACATATTCAGAACTATTT
TAAAAGCCACTTTTGCAGGAAGCCTTTGCTGACTTCTTAACCACCCCCAGAATATCACTGTC

U-PI6_D10 (SEQ ID NO:39)

GTACACAACAGCTTTATTGAGCCCCGGCTGGGTGGTGCTTCCTCCTCCATGTTAGAACCTCCCTGTGTT
GGCCATGGGCACCCCCCAAGCTCTGCCCGTCAATTCCTACAAACTGCTCATCTCTAGCATCAGTGCCTC
CCAAGGATTGGGGGTGCTTCTCCTGCTAGTGATGATGGCCGCCATCTTGGGGGGGATGGGATGAGGGCT
GGGGCGTGTCACCTCAGGTGAGGTGGAAGAGCTGGATGAGGGTGTTGGTCAAGAAGAAAAGGATGCTGC
CACCGGCCAGGCTGCCGAAGGCTGGGGAGCCACTTCGGGGTTTCTTGGAGGTGCTGATGGTATGGTTTC
CCAGGACTCCAGTTTGTATCTGAATCATAATCAGGAGGCTGATGGTGAAGAGGAGGAAGAGGAAGCCTT
TCATTTTGGGAGGCTCTCCGCGGCTCTCTTGGTAGCAGCTTTTGGAGATGAACTCAGGGCTGTCTGCCC
CGCGTACCTCGGCCGCGACCACGCTGGG

U-PI6_E03 (SEQ ID NO:40)

GTACTGTAATTTTTAAAGACTTTTAAAGGTAGTTGCTAACTAATTTCTTTTTTCTTTGATTCACAGAAG
GATGAAGATTCTGCAGTGACTGTATCTGTGGTGATTGTGCAGATTTTTCATGAAAGAGAGAATAAACTA
AGACCTTTTACAAAAAAAAAAAAAAAAAAAAAA

U-PI6_F10 (SEQ ID NO:41)

GTACGCGGGACGTTTAGTCTCATTGCACTGTGTTCTTTACCACTAGAACCAATCTGGGAAACCTGAAGA
CTAGTATACAACAACTCAAACACATTAAATCACGCCCCCATGGACACATAAAAGTTTAATTCCAAGTTA
AACTATCTAGAAAATTAATATGAAGGTCAAAATGGGACAGTTTTTCTAGTTGAAAACCCTGTTTATTTA
TTCTTTAATCCAAACATTTTTACAAATCTGAAAAAGAGGTTGATTACATTTTAAATATTCACTTCTGCC
TATTTGGTTCTGTTTGGTTAAAGGGTGATGGAATTTGGCCAGGC

U-PI6_G06 (SEQ ID NO:42)

GTACAGCTGGGTTCTTTTCATCTGAGACCCCTTCCACCTTAGTGCCAAGACTCCTCATGGCTGTCGTCT
CCAGACCTCCAGAACCCAGACTCTGAAAGGGGACTCCAGCCTTTAAGAGGCTTTCAGATTGATGCTTTA
CCCCTGCCCTTGACAACTGGGACAGAAGCGAACATGTGCCTGTGGCCATTAAGAAGCCAATTCCCTGCA
GACAGTAGTTCAGGGAAGCAGGGAGCAGAGAGAGGCTGCACATCCCGTGAACTGGAGAGGAAGGACTAT
GTCAAAACCTGAGAGTCGAGGTGCCCAGCAAAGGAAGCCTGACCTACTCTTAGCAGTCAAGTTCTGCCT
CACCCTTGG

U-PI6_G08 (SEQ ID NO:43)

GTACACGCTGACAGAGTGCCTCCATCTTCAGCGAGGATCACTGTGTTTCAGTTCAGTGATGGCATAAAG
CACTAATTACTGACTTCTAATCCAACTCTAACAGCCTGCATCAAGACAGCAGAATTAGCCACAGGTATC
AAAGTATTTAAACCAGTATTCTGTGATTGTTTTCTTGGACGCAGGAAAATTCCAGGGTTAGGCTAGTTT
TAAGTGAAAAAGCCACAGGATAAAAAAAGGGTTCACCAGGGAAAAATACACACAAACAAACCCACACAT
CAACCAACCAATCAGCAACTGGTAAGATTTGTTATTGGCCAGGCTGACT

U-PI6_G11 (SEQ ID NO:44)

GTACGCGGGTGGAGGCCTTTGCAGAGAAAGCAAACTGAGCCCCAGTGAGGATTATAAATTGGTTAGACA
AATAGGAGGAGAAATATGGAAAGTAAAATGCTCAGGCACATTGAGGAGAAGTTGGTGCTAAGAACAGAG
CTGAAGCAAGAAGAGGCAGGAGCCAGGTCAAGCAGAGGTATTTAGGCACTGCCCAAGTCTTATATACTT
GCCCATCCCTGCCTCTTGCTGCCTTCTGTTCCCCCCACAACTCTCATCCCTACATATTCAGAACTATTT
TAAAAGCCACTTTTGCAGGAAGCCTTTGCTGACTTCTTAACCACCCCCAGAATATCACTGTC

U-PI6_H02 (SEQ ID NO:45)

GTACTTGACAACATGTCCAAGAGTGACATAGGTAGAATTTAACATTTTTAATTTATTCTTGTCCTCCCA
ACAAAAAAGAATTTCAAATGGTGACATGAATATTTTTTTCTTTCTCTCCACACTTCCTAATTGCCCTTA
ATATGGAGTTAAATTATCCTTATATGTGTAATTAAGCATTTGTTGTATTTGTTATTAATAATATTTACT
GTCTCCATGGTTAATTAGGGAGTTAAATAGGAAAAAATATATGGTACCTGCCCTGGCGGCCGCTCGAGG
G

U-PI6_H10 (SEQ ID NO:46)

GTACAGTTTAAGATAACTGAATTATGGATAATTGTATTTGATCTCTTCACTAGTATTCTCAATTGAGTG
ACCATACAAGTGAAACCATTCAAATTTAGATTTGGGGAATGGTAAAGGAATCAACTTTTTATATTGCTT
GTTGGGGGAAAATACTGAAGATTATCATTTCATGGGTCAGCAGATTGACTTGTTAAGATGGTAAATCAG
ATCATAAGCATCCAGTTAAAGACATACAGGGGTTGAAAACTATTTGAATCTTTGAGATGTTATTGCCCT
AGAGACAGCTAAATCAGTACCTGCCCTGGCGGCCGCTCGAGGG

Discussion

Development of a BVDV Diagnostic Test

Clones found to be specific to blood cells in persistently infected animals, or acutely infected animals are used in RNA- or protein-based diagnostics.

An RNA diagnostic may entail reverse transcriptase polymerase chain reaction approaches. These approaches enable veterinarians or producers to perform the procedure out in the field or on the farm.

Protein diagnostics are currently the most common type of field diagnostic test. In the instant invention, in cases where a surrogate marker is secreted, it will be possible to use the coding region of the BVDV-specific cDNA and to express recombinant protein using one of a standard protein expression system (E. coli, yeast, baculovirus, and the like).

In order to make an appropriate ELISA, the protein or peptide in question is modeled via protein structure software to identify a region that would have a high probably of being exposed on the outside of the protein (as opposed to a region that might be buried in a hydrophobic pocket). Polyclonal antibody is made against this peptide antigen and used as the "capture" antibody. In a preferred embodiment, the antibody is detectably

labeled. A lateral flow system is developed where polyclonal anti-peptide antibody coupled to antigen in the blood flow over a polyclonal antibody against the entire recombinant protein to localize the detectable signal on a solid support-membrane. This type of test is desirable, because it is user friendly and does not require a great deal of technical expertise and equipment. Monoclonal antibodies may be generated to improve specificity. Also if the antigen is an intracellular protein, a method to lyse the cells may be added. Any antigen found in the blood also may be expressed in other tissues of the infected animals. For example, an immunohistochemical approach using an ear notch or some other easily accessible sample may be used.

Thus herein is provided a simple and convenient method of differentially diagnosing persistent infection BVDV from acute BVDV and acute BVDV from vaccinated animals, based on comparison of secondary markers derived from subtraction libraries.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.